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## Endosymbiotic microorganisms of aphids (Hemiptera: Sternorrhyncha: Aphidoidea): Ultrastructure, distribution and transovarial transmission

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**Key words.** Aphidoidea, endosymbiotic bacteria, bacteriocytes, transovarial transmission of endosymbiotic bacteria

**Abstract.** The ultrastructure, distribution and transovarial transmission of endosymbiotic bacteria in representatives of six aphid families: Eriosomatidae (*Pemphigus spyrothecae*, *Prociphilus fraxini*), Anoeciidae [*Anoecia (Anoecia) corni*], Drepanosiphidae [*Mindarus abietinus*, *Sipha (Rungsia) maydis*, *Clethrobium comes*, *Myzocallis (Lineomyzocallis) walshii*], Thelaxidae (*Thelaxes dryophila*), Aphididae (*Delphinobium junackianum*, *Aphis viburni*, *Cavariella theobaldi*, *Macrosiphoniella tanacetaria*) and Lachnidae (*Schizolachnus pineti*, *Eulachnus rileyi*) were studied at the ultrastructural level. The ovaries of aphids are accompanied by large organs termed bacteriomes that consist of giant cells termed bacteriocytes. The bacteriocyte cytoplasm is tightly packed with endosymbiotic bacteria. Ultrastructural observations have shown that the bacteria *Buchnera aphidicola* (primary symbiont of aphids) present in various species are characterized by significant differences in both size and organization of their cytoplasm. In the aphids, *Prociphilus fraxini*, *Sipha (Rungsia) maydis*, *Thelaxes dryophila*, *Aphis viburni*, *Cavariella theobaldi*, *Macrosiphoniella tanacetaria*, *Eulachnus rileyi* and *Schizolachnus pineti*, in addition to *Buchnera aphidicola*, secondary endosymbionts are also present. The bacteriocytes containing secondary endosymbionts are less numerous than those with *Buchnera*. In *Eulachnus rileyi* (Lachnidae), in addition to primary and secondary endosymbionts, there is a third type of microorganism. In all species examined both the primary and secondary endosymbionts are transovarially transmitted from mother to offspring.

### INTRODUCTION

Many insects live in symbiotic associations with prokaryotic (i.e. bacteria) or eukaryotic (i.e. yeasts) microorganisms. Numerous extensive studies have revealed that endosymbiotic microorganisms may occur in the body of a host insect extracellularly (e.g. in the gut lumen) or intracellularly (in specialized cells of mesodermal origin termed bacteriocytes or mycetocytes) (see Buchner, 1965; Douglas, 1989, 1998; Moran & Baumann, 2000; Ishikawa, 2003; Baumann, 2005; Kikuchi, 2009 for further details). Bacteriocytes contain bacteria, whereas mycetocytes harbour yeasts. Both bacteriocytes and mycetocytes are large, polyploid cells that as a rule are integrated into large organs termed bacteriomes or mycetomes, respectively. Buchner (1965) postulated that in insects two categories of endosymbionts may be present: obligate ones (currently termed primary endosymbionts or P-symbionts) and accessory ones (currently termed facultative endosymbionts, secondary endosymbionts or S-symbionts). The primary endosymbionts occur in all specimens of a given species and are always transovarially (i.e. vertically, maternally) transmitted from one generation to the next. The occurrence of primary endosymbiotic microorganisms in the body of plant sap-sucking hemipterans is connected with their diet, which is deficient in amino acids (Douglas, 1989, 1998; Douglas & Prosser, 1992; Lai et al., 1994; Sasaki & Ishikawa, 1995; Baumann et al., 1995). The presence of primary endosymbionts seems to be indispensable since they are necessary

for both survival and reproduction of host insects. In contrast to primary endosymbionts, secondary ones may occur not only within bacteriocytes but also in other cells (e.g. fat body cells) or even free in the haemolymph (Moran & Telang, 1998; Fukatsu et al., 2000; Oliver et al., 2006, 2010). The secondary endosymbionts are, as a rule, only in individuals of some subpopulations. The role of secondary endosymbionts is still unclear, but results of recent studies indicate that aphids that possess secondary endosymbionts are more resistant to heat stress (Montlor et al., 2002), attack by parasitic hymenopterans (Oliver et al., 2003; Vorburger et al., 2010) and infection by fungal pathogens (Scarborough et al., 2005) than those that lack them. It is also suggested that secondary endosymbionts may compensate for the loss of the primary endosymbiont of aphids, the bacterium *Buchnera* (see below) and are able to supplement insufficient provisioning of amino acids usually provided by *Buchnera* (Koga et al., 2003; Burke et al., 2009). The secondary endosymbionts may be transmitted either vertically or horizontally (Buchner, 1965).

It is believed that associations between insects and primary endosymbionts are results of single, ancient infections (Buchner, 1965). Analysis of 16S rDNA sequences of the primary endosymbiont of aphids, *Buchnera aphidicola*, revealed that the association between aphids and their primary endosymbiont resulted from an infection that occurred 200 to 250 million years ago (Unterman et al., 1989; Moran & Baumann, 1994; Martinez-Torres et

TABLE 1. List of the species studied.

Species	Family	Place of collection	Date of collection	Host plant
<i>Pemphigus spyrothecae</i>	Eriosomatidae	Katowice	May, 2007, 2008	<i>Populus nigra</i>
		Katowice	October, 2008	<i>Populus nigra</i>
<i>Prociphilus fraxini</i>	Eriosomatidae	Gliwice, Cracow	April, May, 2008 October, 2009	<i>Fraxinus excelsior</i>
<i>Anoecia (Anoecia) corni</i>	Anoeciidae	Ojcow, Katowice, Cracow	August, 2004 June, 2007 October, 2006, 2010	<i>Cornus sanguinea</i>
<i>Mindarus abietinus</i>	Drepanosiphidae	Ojcow, Gorce, Myslenice	June, 2007, 2009, 2010	<i>Abies alba</i>
<i>Sipha (Rungsia) maydis</i>	Drepanosiphidae	Zawada near Czestochowa Dobrowa Gornicza	September, October, 2009	<i>Arrhenatherum elatius</i>
<i>Clethrobisus comes</i>	Drepanosiphidae	Katowice	April, August, 2009 October, 2008, 2009	<i>Alnus incana</i>
<i>Myzocallis (Lineomyzocallis) walshii</i>	Drepanosiphidae	Katowice	October, 2005	<i>Quercus rubra</i>
<i>Thelaxes dryophila</i>	Thelaxidae	Katowice	May, 2007	<i>Quercus robur</i>
<i>Delphiniobium junackianum</i>	Aphididae	Ojcow	May, September, 2005	<i>Aconitum firmum</i>
<i>Aphis viburni</i>	Aphididae	Kasna Gorna near Tarnow Ojcow,	June, October, 2006, 2010	<i>Viburnum opulus</i>
<i>Cavariella theobaldi</i>	Aphididae	Kotlina Nowotarska	May, September, 2003	<i>Heracleum sphondylium</i>
<i>Macrosiphoniella tanacetaria</i>	Aphididae	Borne-Sulimowo near Szczecinek	June, 2004	<i>Tanacetum vulgare</i>
<i>Schizolachnus pineti</i>	Lachnidae	Bochnia	July, October, 2009, 2010	<i>Pinus sylvestris</i>
<i>Eulachnus rileyi</i>	Lachnidae	Ustron	May, September, 2009	<i>Pinus sylvestris</i>

al., 2001; Moran et al., 2008). In contrast to primary endosymbionts, the associations between insects and secondary endosymbionts are results of more recent multiple and independent infections (Moran & Telang, 1998; Sandström et al., 2001; Thao & Baumann, 2004; Baumann, 2006).

Within aphids, only representatives of the family Phylloxeridae (superfamily Phylloxeroidea) do not contain endosymbiotic microorganisms (Buchner, 1965; Szklarzewicz et al., 2009). Aphids belonging to the family Adelgidae (superfamily Adelgoidea) may harbour gamma- or/and betaproteobacterial endosymbionts (Toenshoff et al., 2012a, b; von Dohlen et al., 2013). In aphids belonging to the superfamily Aphidoidea (except for some representatives of the family Hormaphididae) a gammaproteobacterium, *Buchnera aphidicola*, is present (see Baumann et al., 1995; Douglas, 2003 for further details).

Results of molecular analyses have revealed that during the co-evolution between the bacterium *Buchnera aphidicola* and aphids, the bacterial genome underwent a process of reduction, which resulted in the loss of some genes, e.g. those controlling the synthesis of nonessential amino acids, sterols and cell wall components, and controlling regulation and cell defense (see Ishikawa, 2003; Latorre & Moya, 2006 for further details). As a consequence, (1) the genome of *B. aphidicola* is one seventh of that of its close relative, *Escherichia coli* and (2) these endosymbionts are not able to live outside their host (Charles & Ishikawa, 1999). In contrast to genes for the

synthesis of nonessential amino acids that have been lost, there are multiple copies of the genes responsible for the synthesis of essential amino acids in the genome of *B. aphidicola* (Lai et al., 1994). Since the bacteria *B. aphidicola* living in different species of aphids evolved independently from each other (i.e. without gene exchange between bacteria in different hosts), the comparison of gene sequences (mainly 16S rDNA) of these endosymbionts appeared to be very useful to construct the phylogenetic tree of aphids (Clark et al., 2000; Normark, 2000; Martinez-Torres et al., 2001; Hypša & Nováková, 2009; Jouselin et al., 2009; Perez-Brocal et al., 2011; Liu et al., 2013; Nováková et al., 2013).

Although endosymbiotic microorganisms of aphids have been extensively studied, there are, however, very few studies of their ultrastructure, localization in the host's body and mode of transmission to the next generation. This fact prompted us to investigate aspects of symbiosis listed above both in viviparous and oviparous generations of six families of aphids belonging to the superfamily Aphidoidea.

## MATERIAL AND METHODS

### Insects

Viviparous and oviparous females of 14 species belonging to six families of the Aphidoidea were examined: Eriosomatidae [*Pemphigus spyrothecae* Passerini, 1856; *Prociphilus fraxini* (Fabricius, 1777)], Anoeciidae [*Anoecia (Anoecia) corni* (Fabricius, 1775)], Drepanosiphidae [*Mindarus abietinus* Koch, 1857; *Sipha (Rungsia) maydis* Passerini, 1860; *Clethrobisus comes* (Walker, 1848); *Myzocallis (Lineomyzocallis) walshii* (Monell

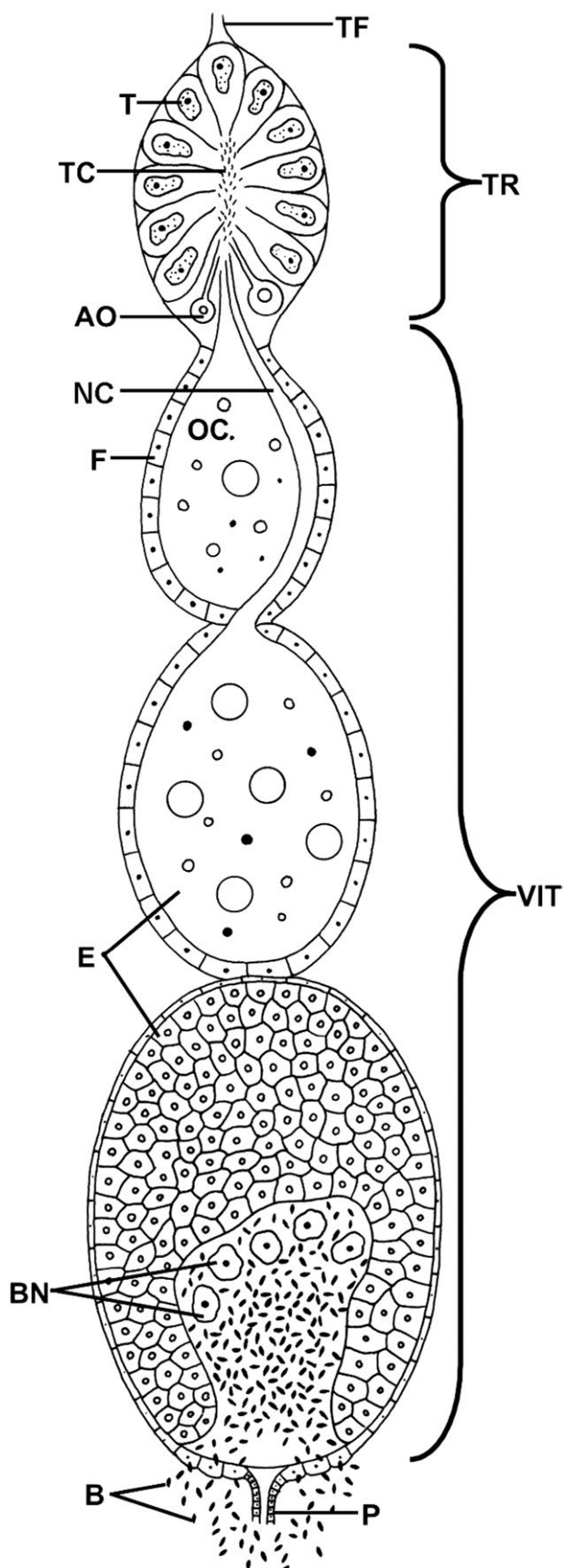


Fig. 1. Schematic representation of the structure of an ovary and transovarial transmission of endosymbiotic bacteria in viviparous generations of aphids. Abbreviations: AO – arrested oocyte, B – endosymbiotic bacteria, BN – bacteriocyte nuclei, E – embryo, F – follicular epithelium, NC – nutritive cord, OC – oocyte, P – pedicel, T – trophocyte, TC – trophic core, TF – terminal filament, TR – trophiarium, VIT – vitellarium

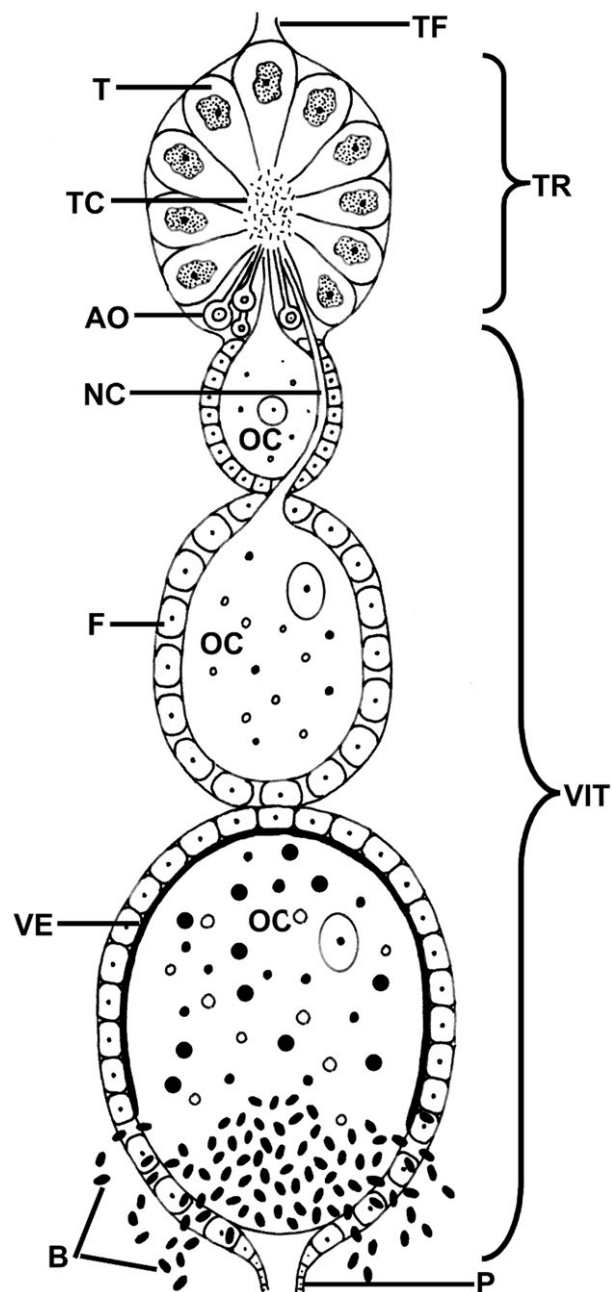
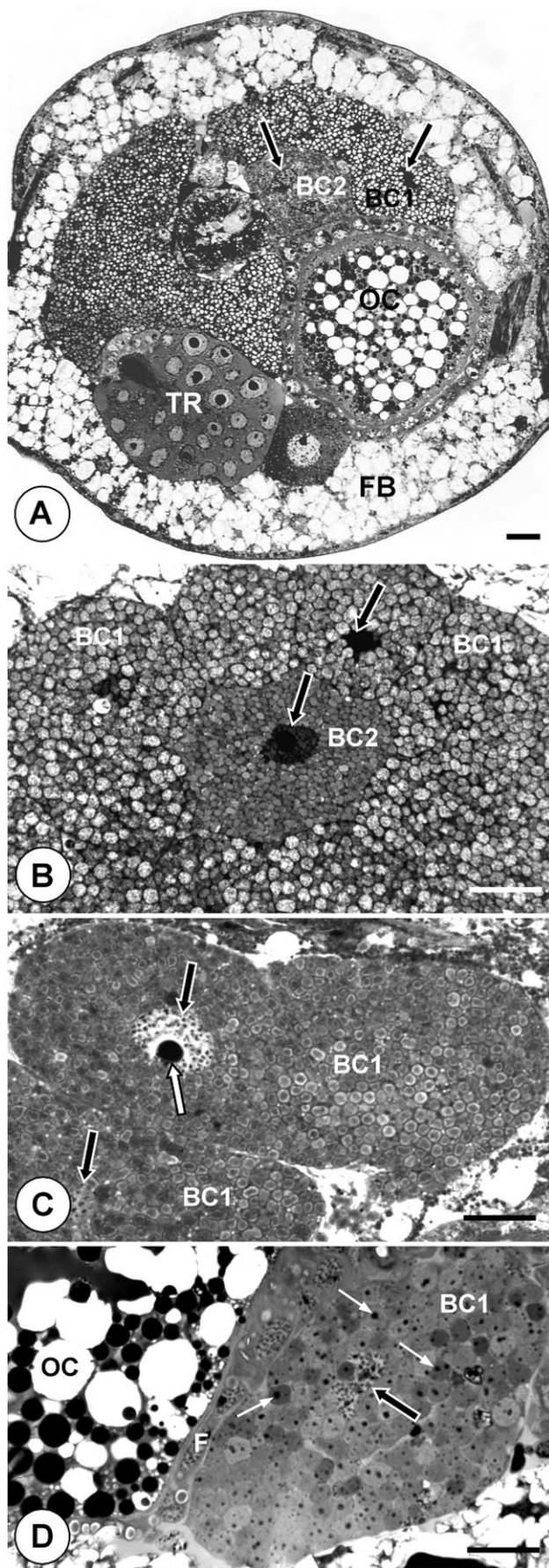


Fig. 2. Schematic representation of the structure of an ovary and transovarial transmission of endosymbiotic bacteria in oviparous generation of aphids. Abbreviations: AO – arrested oocyte, B – endosymbiotic bacteria, F – follicular epithelium, NC – nutritive cord, OC – oocyte, P – pedicel, T – trophocyte, TC – trophic core, TF – terminal filament, TR – trophiarium, VIT – vitellarium, VE – egg envelopes.

in Riley & Monell, 1879)], Thelaxidae [*Thelaxes dryophila* (Schrank, 1801)], Aphididae [*Delphiniobium junackianum* (Karsch, 1887); *Aphis viburni* Scopoli, 1763; *Cavariella theobaldi* (Gillette & Bragg, 1918); *Macrosiphoniella tanacetaria* (Kaltenbach, 1843)], Lachnidae [*Schizolachnus pineti* (Fabricius, 1781); *Eulachnus rileyi* (Williams, 1911)]. All species were collected in the south of Poland. For each species ten specimens were examined.

Place, date of collection and the host plant of the species studied are summarized in Table 1.



### Light and electron microscopy (TEM)

Females of all species studied were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Next, the material was rinsed in a mixture of the buffer and sucrose (5.8 g/100 ml) and postfixed in 1% osmium tetroxide in the same buffer. After dehydration in a graded series of ethanol and acetone, the material was embedded in epoxy resin Epon 812 (Serva, Heidelberg, Germany). Semithin sections were stained with 1% methylene blue in 1% borax, examined and photographed using Leica DMR and Nikon Eclipse 80i light microscopes. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined and photographed using a JEM 100 SX electron microscope at 80 kV.

### RESULTS

#### Gross morphology of the ovary

The ovaries of aphids consist of several telotrophic ovarioles (Figs 1, 2). An individual ovariole is composed of four well-defined regions: a terminal filament, tropharium (trophic chamber), vitellarium and pedicel (ovariolar stalk) (Figs 1, 2). The latter joins the ovariole to the lateral oviduct. The tropharium consists of individual trophocytes and early previtellogenic oocytes termed arrested oocytes. The central part of the tropharium is occupied by a cell-free area termed the trophic core (Figs 1, 2). The vitellarium contains linearly arranged ovarian follicles (Figs 1, 2). Each follicle encloses a developing oocyte surrounded by a one-layered follicular epithelium (Figs 1, 2). All oocytes (arrested and those in the vitellarium) are connected to the trophic core by nutritive cords (Figs 1, 2). In oviparous females oocytes develop through three stages: previtellogenesis, vitellogenesis and choriogenesis (Fig. 2). During previtellogenic growth RNAs (synthesized by trophocytes) are transported along the nutritive cord into the cytoplasm of the oocyte. During vitellogenesis the volume of the oocyte increases due to the accumulation of reserve substances (i.e. yolk granules and lipid droplets) in its cytoplasm. During choriogenesis, as a result of the

Fig. 3. Distribution of endosymbiotic microorganisms. A – *Anoezia* (*Anoezia*) *corni* (Anoeziidae). Oviparous generation. Cross section of the abdomen. In the body cavity of a mature female bacteriocytes with endosymbiotic microorganisms fill the spaces between internal organs. Abbrev.: BC1 – bacteriocyte with bacteria *Buchnera aphidicola* (primary endosymbiont), BC2 – bacteriocyte with secondary endosymbionts, FB – fat body, OC – oocyte, TR – tropharium, black arrow – bacteriocyte nucleus. Methylene blue, scale bar – 20 μm. B – *Anoezia* (*Anoezia*) *corni* (Anoeziidae). Oviparous generation. Bacteriome. Abbrev.: BC1 – bacteriocyte with *Buchnera aphidicola*. BC2 – bacteriocyte with secondary endosymbionts, black arrow – bacteriocyte nucleus. Methylene blue, scale bar – 20 μm. C – *Thelaxes dryophila* (Thelaxidae). Viviparous generation. Bacteriocytes with *Buchnera aphidicola* (BC1), black arrow – bacteriocyte nucleus, white arrow – bacteriocyte nucleolus. Methylene blue, scale bar – 20 μm. D – *Prociphilus fraxini* (Eriosomatidae). Oviparous generation. Fragment of the bacteriocyte with *Buchnera aphidicola* (BC1). Abbrev.: OC – oocyte, F – follicular epithelium, black arrow – bacteriocyte nucleus, white arrows – electron-dense aggregates. Methylene blue, scale bar – 20 μm.



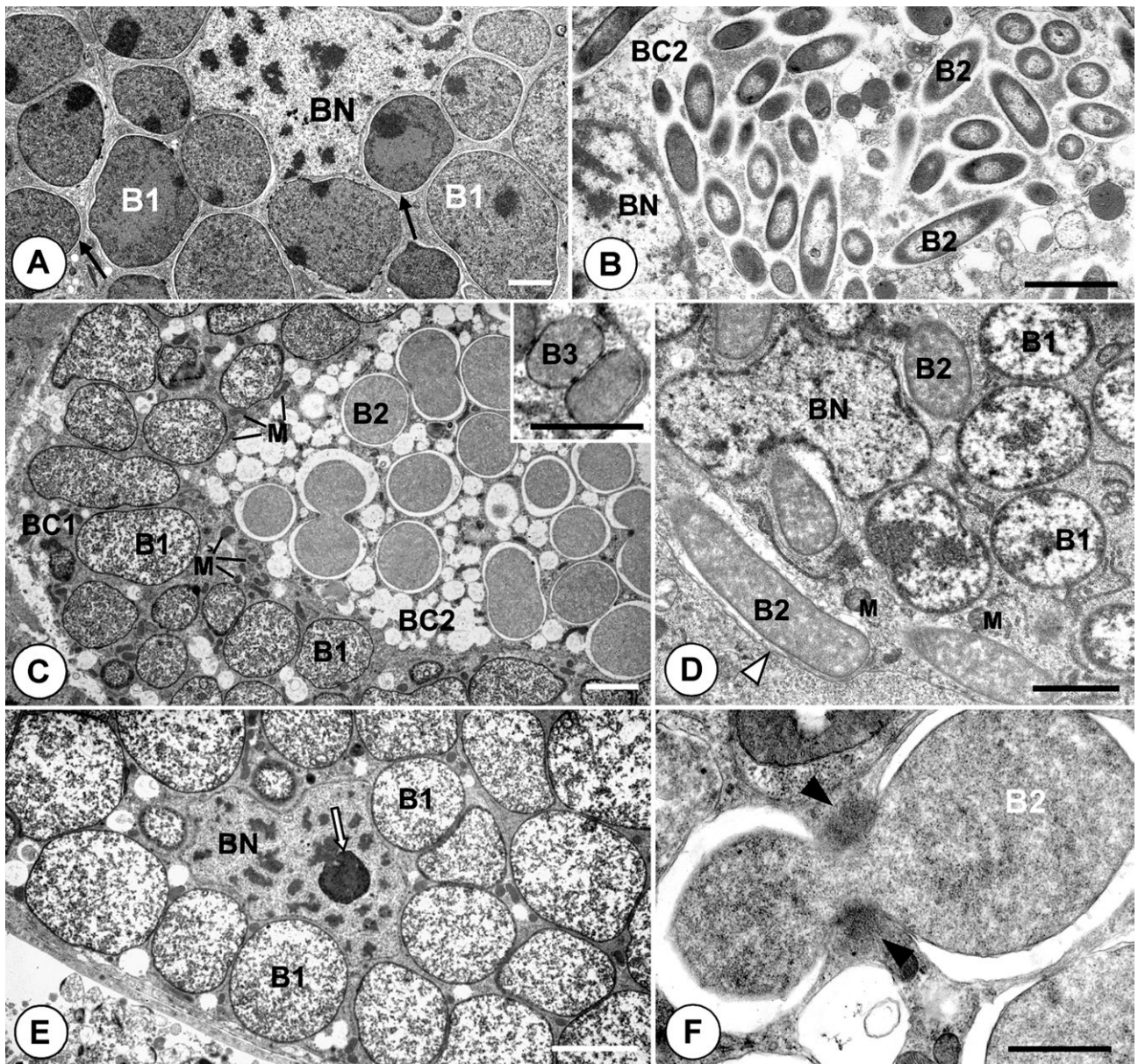


Fig. 4. Ultrastructure of endosymbiotic microorganisms. A – *Prociphilus fraxini* (Eriosomatidae). Oviparous generation. Fragment of the bacteriocyte with bacteria *Buchnera aphidicola*. Abbrev.: B1 – bacterium *Buchnera aphidicola*, BN – bacteriocyte nucleus, black arrow – perisymbiotic membrane. TEM, scale bar – 2 μm. B – *Cavariella theobaldi* (Aphididae). Viviparous generation. Fragment of the bacteriocyte with secondary endosymbionts (BC2). Abbrev.: B2 – secondary endosymbiont, BN – bacteriocyte nucleus. TEM, scale bar – 2 μm. C – *Schizolachnus pineti* (Lachnidae). Viviparous generation. Fragment of the bacteriome containing bacteriocytes with bacteria *Buchnera aphidicola* and bacteriocyte with secondary endosymbionts. Abbrev.: B1 – bacterium *Buchnera aphidicola*, B2 – secondary endosymbiont, BC1 – bacteriocyte with bacteria *Buchnera aphidicola*, BC2 – bacteriocyte with secondary endosymbionts, M – mitochondria. TEM, scale bar – 3 μm. Insert: *Eulachnus rileyi* (Lachnidae). Viviparous generation. Third type of microorganism (B3) in the cytoplasm of the bacteriocyte containing the bacterium *Buchnera aphidicola*. TEM, scale bar – 1 μm. D – *Aphis viburni* (Aphididae). Viviparous generation. Primary and secondary endosymbionts in same bacteriocyte. Abbrev.: B1 – bacterium *Buchnera aphidicola*, B2 – secondary endosymbiont, BN – bacteriocyte nucleus, M – mitochondrion, white arrow-head – bacteriocyte membrane, TEM, scale bar – 2 μm. E – *Pemphigus spyrothecae* (Eriosomatidae). Viviparous generation. Fragment of the bacteriocyte with bacteria *Buchnera aphidicola*. Abbrev.: B1 – bacterium *Buchnera aphidicola*, BN – bacteriocyte nucleus, white arrow – bacteriocyte nucleus. TEM, scale bar – 4 μm. F – *Eulachnus rileyi* (Lachnidae). Viviparous generation. Dividing secondary bacterium (B2); black arrowheads – electron-dense material. TEM, scale bar – 1 μm.

synthetic activity of follicular cells the oocyte becomes covered with egg envelopes (for a detailed description of ovaries of aphids see Büning, 1985; Szklarzewicz et al., 2000, 2009; Michalik et al., 2013). In viviparous females the oocyte growth stops at the stage of previtellogenesis.

Soon after previtellogenesis the nucleus undergoes a series of synchronous divisions resulting in the formation of an embryo (Fig. 1).

TABLE 2. Sizes of primary endosymbiotic bacteria (*Buchnera aphidicola*) in the species of aphids studied ( $\bar{x}$  – mean, S.D. – standard deviation).

No.	Species	Family	Diameter $\bar{x}$ [ $\mu$ m]	N	Min. [ $\mu$ m]	Max. [ $\mu$ m]	S.D.
1.	<i>Pemphigus spyrothecae</i>	Eriosomatidae	4.53	19	4.04	5.33	0.33
2.	<i>Prociphilus fraxini</i>	Eriosomatidae	4.14	19	3.14	5.23	0.62
3.	<i>Anoecia (Anoecia) corni</i>	Anoeciidae	3.19	18	2.33	3.9	0.52
4.	<i>Mindarus abietinus</i>	Drepanosiphidae	3.14	17	2.41	3.5	0.24
5.	<i>Sipha (Rungsia) maydis</i>	Drepanosiphidae	2.48	19	2	3.14	0.32
6.	<i>Clethrobis comes</i>	Drepanosiphidae	3.92	18	3.1	4.8	0.58
7.	<i>Myzocallis (Lineomyzocallis) walshii</i>	Drepanosiphidae	2.58	19	2.25	2.88	0.19
8.	<i>Thelaxes dryophila</i>	Thelaxidae	3.3	19	2.85	4.32	0.32
9.	<i>Delphinobium junackianum</i>	Aphididae	3.2	19	2.57	3.97	0.38
10.	<i>Aphis viburni</i>	Aphididae	2.63	19	2.14	3.45	0.3
11.	<i>Cavariella theobaldi</i>	Aphididae	2.09	16	1.9	2.55	0.2
12.	<i>Macrosiphoniella tanacetaria</i>	Aphididae	2.51	19	2.17	3.11	0.24
13.	<i>Eulachnus rileyi</i>	Lachnidae	2.69	19	2.5	2.92	0.1
14.	<i>Schizolachnus pineti</i>	Lachnidae	3.45	19	2.68	3.97	0.33

### Ultrastructure and distribution of endosymbiotic microorganisms

In the body of both viviparous and oviparous females of all the species of aphids studied large organs termed bacteriomes occur in the close vicinity of the ovaries (Fig. 3A). The bacteriomes consist of numerous giant cells termed bacteriocytes (Fig. 3A, B, C, D). The bacteriocyte cytoplasm is tightly packed with endosymbiotic bacteria (Figs 3A–D, 4A–E). In most aphids there are two types of bacteriocytes: one type contains the bacterium *Buchnera aphidicola* (Fig. 3A–D) and the other secondary symbionts (Fig. 3A, B). Both types of bacteriocytes are usually

spherical with a diameter of about 80  $\mu$ m. The bacteriocytes possess large, spherical or amoeboid nuclei with single nucleoli and aggregations of heterochromatin (Figs 3B, C, 4A, D, E). The bacterium *B. aphidicola* is usually spherical or oval (Figs 3A–C, 4D, E), but may be also amoeboid in shape (Figs 3D, 4A, C). Their diameter ranges from 1.9 to 5.33  $\mu$ m (see Table 2). In representatives of the family Eriosomatidae, the size of *B. aphidicola* is much larger than in the other aphids studied. In most species of aphids the cytoplasm of *B. aphidicola* is not differentiated into distinct zones (Fig. 4 C, E). In the cytoplasm of these bacteria, in some species of aphids (*Prociphilus*

TABLE 3. Sizes of the secondary endosymbiotic bacteria in the species of aphids studied ( $\bar{x}$  – mean, S.D. – standard deviation).

No.	Species	Family	Length $\bar{x}$ [ $\mu$ m]	N	Min. [ $\mu$ m]	Max. [ $\mu$ m]	S.D.	Diameter $\bar{x}$ [ $\mu$ m]	N	Min. [ $\mu$ m]	Max. [ $\mu$ m]	S.D.
1.	<i>Pemphigus spyrothecae</i>	Eriosomatidae	–	–	–	–	–	–	–	–	–	–
2.	<i>Prociphilus fraxini</i>	Eriosomatidae	5.14	15	4.6	5.8	0.29	3.02	16	2.8	3.2	0.14
3.	<i>Anoecia (Anoecia) corni</i>	Anoeciidae	–	–	–	–	–	2.08	17	1.97	2.19	0.06
4.	<i>Mindarus abietinus</i>	Drepanosiphidae	–	–	–	–	–	–	–	–	–	–
5.	<i>Sipha (Rungsia) maydis</i>	Drepanosiphidae	7.56	17	6.3	8.45	0.63	1.51	17	1.39	1.7	0.08
6.	<i>Clethrobis comes</i>	Drepanosiphidae	–	–	–	–	–	–	–	–	–	–
7.	<i>Myzocallis (Lineomyzocallis) walshii</i>	Drepanosiphidae	–	–	–	–	–	–	–	–	–	–
8.	<i>Thelaxes dryophila</i>	Thelaxidae	–	–	–	–	–	2.51	17	2.17	3.3	0.32
9.	<i>Delphinobium junackianum</i>	Aphididae	–	–	–	–	–	–	–	–	–	–
10.	<i>Aphis viburni</i>	Aphididae	4.91	17	3.85	5.78	0.5	0.58	17	0.47	0.66	0.05
11.	<i>Cavariella theobaldi</i>	Aphididae	3.56	17	3	4.09	0.33	0.66	17	0.52	0.85	0.11
12.	<i>Macrosiphoniella tanacetaria</i>	Aphididae	7.01	17	6.3	7.8	0.4	1	17	0.7	1.2	0.16
13.	<i>Eulachnus rileyi</i> secondary endosymbionts 1	Lachnidae	–	–	–	–	–	2.3	17	2	2.8	0.21
	<i>Eulachnus rileyi</i> secondary endosymbionts 2	Lachnidae	1.1	6	0.9	1.3	0.14	0.41	6	0.39	0.43	0.014
14.	<i>Schizolachnus pineti</i>	Lachnidae	–	–	–	–	–	3.55	17	3	4.17	0.31

TABLE 4. Types and localization of additional microorganisms (secondary symbionts and a third type of bacteria) in the aphids studied.

No.	Species	Family	Types of additional microorganisms	Localization of additional microorganisms
1.	<i>Pemphigus spyrothecae</i>	Eriosomatidae	absent	—
2.	<i>Prociphilus fraxini</i>	Eriosomatidae	rod-shaped secondary endosymbionts	in separate bacteriocytes
3.	<i>Anoecia (Anoecia) corni</i>	Anoeciidae	spherical secondary endosymbionts	in separate bacteriocytes
4.	<i>Mindarus abietinus</i>	Drepanosiphidae	absent	—
5.	<i>Sipha (Rungsia) maydis</i>	Drepanosiphidae	rod-shaped secondary endosymbionts	in separate bacteriocytes
6.	<i>Clethrobium comes</i>	Drepanosiphidae	absent	—
7.	<i>Myzocallis (Lineomyzocallis) walshii</i>	Drepanosiphidae	absent	—
8.	<i>Thelaxes dryophila</i>	Thelaxidae	spherical secondary endosymbionts	in separate bacteriocytes
9.	<i>Delphinobium junackianum</i>	Aphididae	absent	—
10.	<i>Aphis viburni</i>	Aphididae	rod-shaped secondary endosymbionts	in separate bacteriocytes and bacteriocytes with <i>Buchnera aphidicola</i>
11.	<i>Cavariella theobaldi</i>	Aphididae	rod-shaped secondary endosymbionts	in separate bacteriocytes
12.	<i>Macrosiphoniella tanacetaria</i>	Aphididae	rod-shaped secondary endosymbionts	in separate bacteriocytes
13.	<i>Eulachnus rileyi</i>	Lachnidae	spherical secondary endosymbionts	in separate bacteriocytes
			small, rod-shaped bacteria	in bacteriocytes with <i>Buchnera aphidicola</i>
14.	<i>Schizolachnus pineti</i>	Lachnidae	spherical secondary endosymbionts	in separate bacteriocytes

*fraxini*, *Aphis viburni*), there are electron-dense aggregates (Figs 3D, 4A, D).

In most of the species studied, in addition to *B. aphidicola*, there are secondary endosymbionts. As a rule they are localized in separate bacteriocytes, which are less numerous than those containing *B. aphidicola* (Figs 3A, B, 4B). There is one exception to this rule, *Aphis viburni* (Aphididae). In this species secondary endosymbionts occur both in their own bacteriocytes, but also in the bacteriocytes containing *B. aphidicola* (Fig. 4D).

In the aphids *Anoecia (Anoecia) corni* (Anoeciidae), *Schizolachnus pineti* (Lachnidae) and *Eulachnus rileyi* (Lachnidae) bacteriocytes with secondary endosymbionts are surrounded by those containing *B. aphidicola* (Fig. 3A, B, 4C). Secondary endosymbionts are characterized by various shapes. In the aphids *Anoecia (Anoecia) corni* (Anoeciidae), *Eulachnus rileyi* (Lachnidae), *Schizolachnus pineti* (Lachnidae) and *Thelaxes dryophila* (Thelaxidae) they are spherical and measure from 1.97 to 4.17  $\mu\text{m}$  in diameter. These bacteria are characterized by dark, more homogeneous cytoplasm than that of *B. aphidicola* (Fig. 4C, F). Secondary endosymbionts of *Aphis viburni* (Aphididae), *Cavariella theobaldi* (Aphididae), *Macrosiphoniella tanacetaria* (Aphididae), *Sipha (Rungsia) maydis* (Drepanosiphidae) and *Prociphilus fraxini* (Eriosomatidae) are rod-shaped (Fig. 4B, D). They measure from 3 to 8.45  $\mu\text{m}$  in length and from 0.47 to 3.2

$\mu\text{m}$  in diameter (Table 3). In the body of *Delphinobium junackianum* (Aphididae), *Clethrobium comes* (Drepanosiphidae), *Myzocallis (Lineomyzocallis) walshii* (Drepanosiphidae) and *Pemphigus spyrothecae* (Eriosomatidae) only *B. aphidicola* is present (Fig. 4E).

In bacteriocytes of *Eulachnus rileyi* (Lachnidae), in addition to the primary and secondary endosymbionts, a third type of microorganism occurs (Fig. 4C insert). They are bacteria and occur in the same bacteriocytes as *B. aphidicola* but are significantly less numerous than *B. aphidicola*. They are ovoid in shape, 1.1  $\mu\text{m}$  long and 0.41  $\mu\text{m}$  in diameter (see Table 3). The data on these additional microorganisms in the aphids studied are summarized in Table 4.

Ultrastructural observations indicate that endosymbionts are enclosed by three membranes: two are their own membranes and the third, outer (perisymbiotic) membrane is derived from the host (Fig. 4A).

Both in the bacteriocytes and oocytes all the endosymbiotic microorganisms reproduce by binary fission (Figs 4 C, F, 6F insert).

#### Transovarial transmission of endosymbiotic microorganisms

Both *B. aphidicola* and the secondary endosymbionts in the species of aphids studied are transovarially transmitted from one generation to the next. The moment of



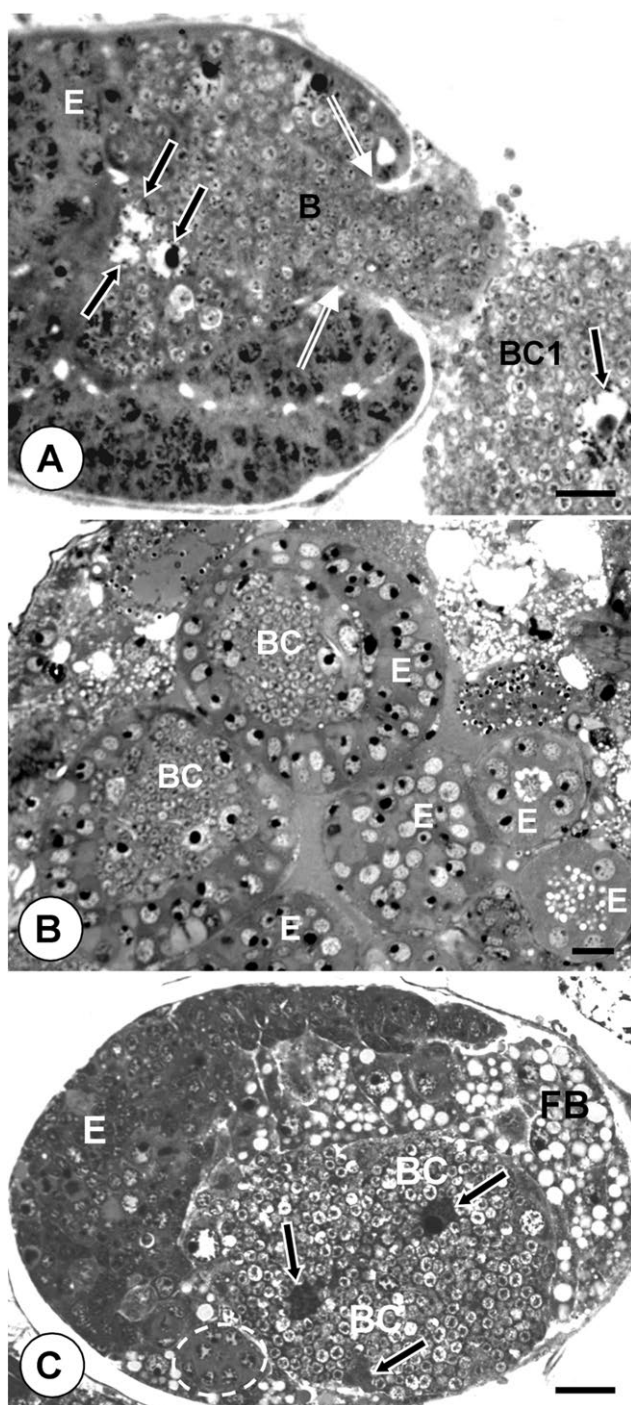


Fig. 5. Transovarial transmission of endosymbionts in viviparous generations. A – *Macrosiphoniella tanacetaria* (Aphididae). Viviparous generation. Bacteria infest the young embryo. Abbrev.: E – embryo, black arrow – bacteriocyte nucleus, B – endosymbiotic bacteria, BC1 – bacteriocyte with *Buchnera aphidicola*, double arrows indicate the opening at the posterior pole of the oocyte. Methylene blue, scale bar – 20  $\mu$ m. B – *Aphis viburni* (Aphididae). Viviparous generation. Young embryos developing in the body cavity of a female. Abbrev.: E – embryo, BC – bacteriocyte with endosymbiotic bacteria. Methylene blue, scale bar – 20  $\mu$ m. C – *Thelaxes dryophila* (Thelaxidae). Viviparous generation. Longitudinal section through a young embryo. Abbrev.: BC – bacteriocyte with endosymbiotic bacteria, E – embryo, FB – fat body, black arrow – bacteriocyte nucleus, encircled by a dotted line – ovary. Methylene blue, scale bar – 20  $\mu$ m.

infestation of embryos (in viviparous females) and oocytes (in oviparous females) is associated with a particular stage of development of the ovaries: in viviparous females bacteria invade ovarioles containing terminal embryos at the blastula stage (Figs 1, 5A), whereas in oviparous females ovarioles containing oocytes are invaded at the stage of choriogenesis (Figs 2, 6B–D). Before invasion, the bacteria leave the cytoplasm of the bacteriocytes.

In viviparous females, follicular cells surrounding the posterior pole of the embryo separate from each other, which facilitates the entry of a huge mass of bacteria into the embryo (Fig. 5A). At the time the bacteria migrate into the embryo, its interior is occupied by a syncytium containing several large nuclei of presumptive bacteriocytes (Fig. 5A). Next, the bacteria invade the cytoplasm of newly formed bacteriocytes (Figs 1, 5B). Initially, the bacteriocytes occupy the posterior pole of the embryo (Fig. 5B, C). In older embryos, the bacteriocytes gather in the vicinity of ovaries and form the bacteriome (Fig. 5C).

In oviparous females, choriogenic oocytes are invaded. Bacteriocytes assemble close to the posterior pole of the terminal oocytes (Fig. 6A). Next, endosymbiotic microorganisms are released from the bacteriocyte cytoplasm into the body cavity. In most aphids endosymbionts migrate to the perivitelline space (= space between oocyte and follicular epithelium) through wide spaces between neighbouring follicular cells (Figs 2, 6B). In the aphids *Clethrobium comes* (Drepanosiphidae) and *Schizolachnus pineti* (Lachnidae), the bacteria migrate both through spaces between neighbouring follicular cells and through the cytoplasm of follicular cells (Fig. 6C, D). From the perivitelline space they migrate into the oocyte cytoplasm (Fig. 6C, D). The entry into the ooplasm is facilitated by the absence of egg envelopes around the posterior pole of the oocyte (Figs 2, 6C, D). When the migration of endosymbionts is completed, follicular cells surrounding the posterior pole of the oocyte start to produce precursors of egg envelopes. Initially, endosymbiotic bacteria accumulate in the cortical cytoplasm at the posterior pole of the oocyte (Fig. 6E). Then they move deeper and form a characteristic “symbiont ball” (Fig. 6F, 6F insert). Endosymbiotic bacteria “wait” in the form of a symbiont ball for the beginning of embryogenesis and the formation of bacteriocytes.

## DISCUSSION

Histological investigations (Kolb, 1963; Buchner, 1965) as well as more recent ultrastructural (e.g. Hinde, 1971; Griffiths & Beck, 1975; Brough & Dixon, 1990; Akhtar & van Emden, 1994; Pyka-Fosciak & Szklarzewicz, 2008, this study) and molecular studies (e.g. Unterman et al., 1989; Baumann et al., 1995; Fukatsu et al., 2000, 2001) have shown that aphids belonging to the superfamily Aphidoidea harbour obligatory endosymbionts. Generally it is considered that the primary symbiont in most aphid families is *Buchnera aphidicola* and that it is the same species in all these aphids (see Introduction) (Munson et al., 1991). An analysis of sequences of the

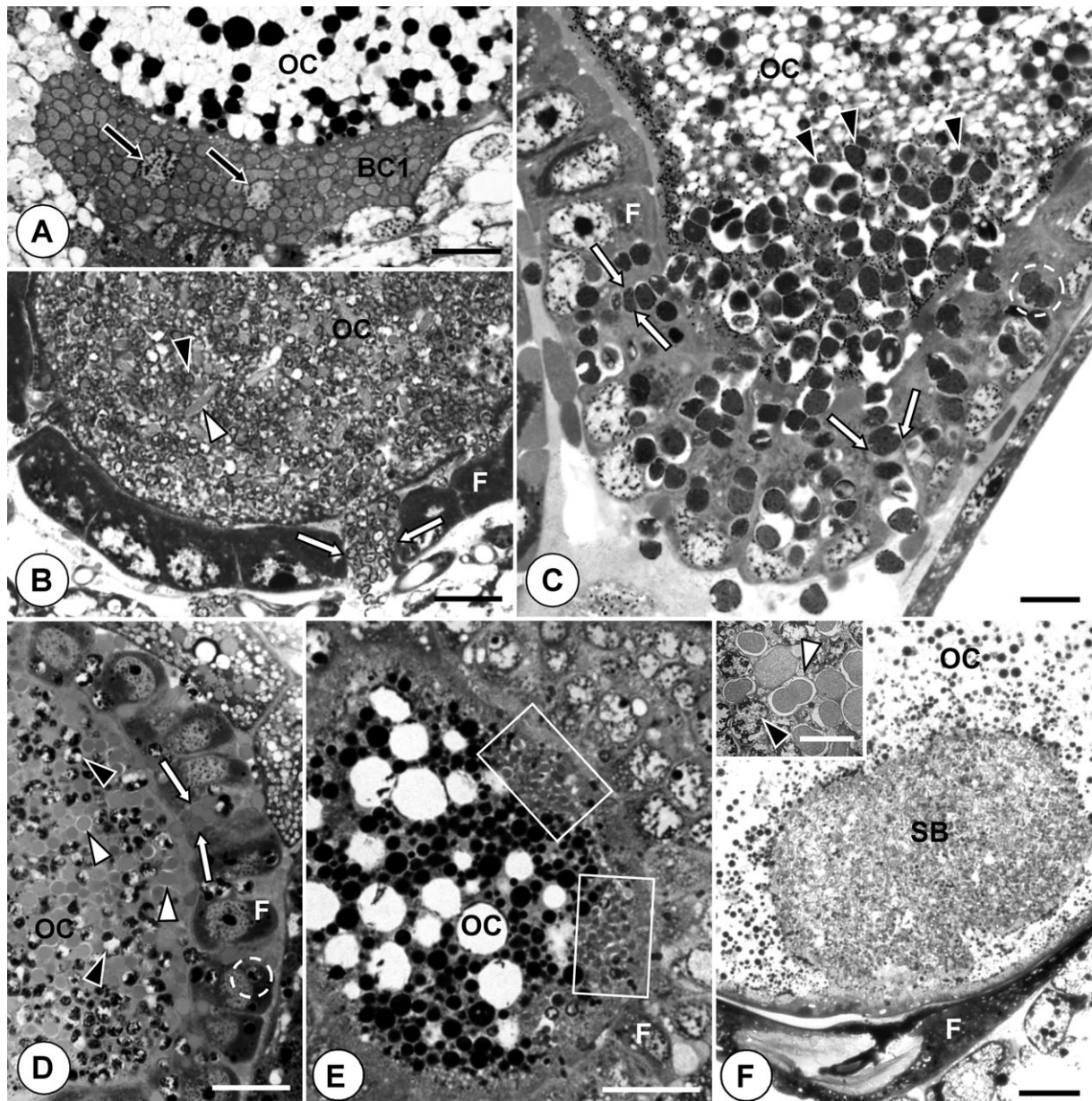


Fig. 6. Transovarial transmission of endosymbionts in oviparous generation. A – *Clethrobium comes* (Drepanosiphidae). Oviparous generation. Bacteriocytes with bacteria *Buchnera aphidicola* close to the posterior pole of the terminal oocyte. Abbrev.: BC1 – bacteriocyte with bacteria *Buchnera aphidicola*, OC – oocyte, black arrow – bacteriocyte nucleus. Methylene blue, scale bar – 20 µm. B – *Sipha (Rungsia) maydis* (Drepanosiphidae). Oviparous generation. Cross section through the posterior pole of the oocyte filled with primary and secondary endosymbionts. Abbrev.: OC – oocyte, F – follicular epithelium, white arrowhead – secondary endosymbiotic bacterium in the oocyte cytoplasm, black arrowhead – bacterium *Buchnera aphidicola* in the oocyte cytoplasm, white arrows indicate endosymbiotic bacteria migrating to the oocyte through the space between neighbouring follicular cells. Methylene blue, scale bar – 20 µm. C – *Clethrobium comes* (Drepanosiphidae). Oviparous generation. Longitudinal section through the posterior pole of the oocyte. *Buchnera aphidicola* bacteria migrating into the cytoplasm of an oocyte. Abbrev.: F – follicular epithelium, OC – oocyte, black arrowheads – the bacterium *Buchnera aphidicola* in the cytoplasm of an oocyte, white arrows – endosymbiotic bacteria migrating into an oocyte through the space between neighbouring follicular cells, encircled – endosymbiotic bacteria migrating into an oocyte through the cytoplasm of a follicular cell. Methylene blue, scale bar – 10 µm. D – *Schizolachnus pineti* (Lachnidae). Oviparous generation. Fragment of the terminal oocyte and follicular epithelium during the migration of endosymbiotic bacteria. Abbrev.: OC – oocyte, F – follicular epithelium, white arrows indicate endosymbiotic bacteria migrating into an oocyte through the space between neighbouring follicular cells, black arrowhead – the bacterium *Buchnera aphidicola* in the cytoplasm of an oocyte, white arrowhead – secondary endosymbiont in the cytoplasm of an oocyte, encircled – endosymbiotic bacteria migrating into an oocyte through the cytoplasm of follicular cell. Methylene blue, scale bar – 20 µm. E – *Anoecia (Anoecia) corni* (Anoeciidae). Oviparous generation. Cross section through the choriogenic oocyte shortly after the entry of endosymbiotic bacteria into the ooplasm. Abbrev.: OC – oocyte, F – follicular epithelium, in frames – endosymbionts in the cortical cytoplasm of an oocyte. Methylene blue, scale bar – 20 µm. F – *Lachnus roboris* (Lachnidae). Oviparous generation. Longitudinal section through an oocyte containing a “symbiont ball” filled with endosymbiotic bacteria. Abbrev.: F – follicular epithelium, OC – oocyte, SB – “symbiont ball”. Methylene blue, scale bar – 20 µm. Insert – *Eulachnus rileyi* (Lachnidae). Oviparous generation. *Buchnera aphidicola* (black arrowhead) and secondary endosymbionts (white arrowhead) in the cytoplasm of an oocyte. TEM, scale bar – 0.5 µm.

16S rRNA gene from *Buchnera* isolated from numerous species of aphids has revealed that there are large differences (up to 17%) in the nucleotide sequence of this gene (data from GeneBank). It is worth noting that Novákova and co-workers (2013) recently analyzed five genes (*groEL*, *trpB*, *dnaB*, *ihvD* and 16S rDNA) of *B. aphidicola* from 70 species of aphids and revealed that there is a great diversity of genomes of this bacterium. Therefore, *Buchnera* from different species of aphids may be subspecies, strains or lineages (e.g. Shigenobu et al., 2000; van Ham et al., 2003; Perez-Brocal et al., 2005; Burke et al., 2009; Novákova et al., 2013). According to Ishikawa (2003), the observed diversification of *Buchnera* indicates that these bacteria can not be considered as representatives of the same species. It should be added that our histological and ultrastructural observations have shown that the bacteria *Buchnera* present in different species of aphids exhibit significant differences in terms of the organization of their cytoplasm and size of their cells. Thus, the differences in their morphology support the conclusions of the molecular analyses.

In many species of aphid, in addition to *B. aphidicola* there are also secondary endosymbionts. Molecular analyses (e.g. Chen et al., 1996; Chen & Purcell, 1997; Fukatsu et al., 2001; Gomez-Valero et al., 2004; Moran et al., 2005; Tsuchida et al., 2005) have revealed that the latter may belong to different taxa. In most species of aphids there are Gram-negative bacteria of the class Gammaproteobacteria of the family Enterobacteriaceae (*Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, *Arsenophonus*), but other representatives of the Alphaproteobacteria (*Rickettsia*, *Wolbachia*) and Gram-positive bacteria (*Spiroplasma*) may be present (see Baumann, 2005; Moran et al., 2008; Oliver et al., 2010 for further details). Moreover, in geographically isolated populations of the same aphid species several different species of secondary endosymbionts may occur (Chen et al., 1996; Chen & Purcell, 1997; Tsuchida et al., 2002). Some of these bacteria (e.g. members of the genus *Arsenophonus*) are present in several species of aphids as well as in other arthropods (Darby et al., 2001; Novákova et al., 2009; Oliver et al., 2010). It should be stressed that geographically isolated populations of the same species of aphid may be devoid of secondary endosymbionts, e.g. specimens of *Cinara cedri* (Lachnidae) from Chile do not possess secondary endosymbionts, while European specimens of this species harbour an obligate secondary symbiont *Serratia symbiotica* (Perez-Brocal et al., 2006; Burke et al., 2009). Previous and present ultrastructural observations indicate that the secondary endosymbionts in the aphids studied always occur in bacteriocytes (Hinde, 1971; Griffiths & Beck, 1975; Brough & Dixon, 1990; Akhtar & van Emden, 1994; Pyka-Fosciak & Szklarzewicz, 2008; this study). As a rule, these bacteria are harboured in their own bacteriocytes. We did not find any secondary endosymbionts free in the haemolymph or in cells of the fat body.

Ultrastructural analyses have revealed that in the body of *Eulachnus rileyi* (Lachnidae), in addition to primary

and secondary endosymbionts, there is a third type of morphologically distinct microorganism. In contrast to primary and secondary endosymbionts, these bacteria do not have own bacteriocytes and are very sparse. It should be noted that three types of endosymbionts are recorded in several other representatives of the family Lachnidae (Klevenhusen, 1927; Buchner, 1965; Gomez-Valero et al., 2004; Pyka-Fosciak, 2006; Pyka-Fosciak & Szklarzewicz, 2008; Burke et al., 2009). The localization of the third type of microorganism in species of lachnid varies, e.g. in *Eulachnus rileyi* they occur together with *Buchnera* (this study), in *Stomaphis quercus* they occur in the same bacteriocytes as the secondary endosymbionts (Pyka-Fosciak & Szklarzewicz, 2006, 2008). The actual function of these bacteria remains unknown. They may represent the evolutionary youngest endosymbionts or may be pathogenic. The size and ultrastructure of these bacteria both in *Eulachnus rileyi* and *Stomaphis quercus* seem to indicate that they may belong to the widespread species infecting arthropods, *Wolbachia pipientis*. This hypothesis is substantiated by the observations of Gomez-Valero and co-workers (2004), who using molecular methods revealed that the third type of microorganism present in the body of *Cinara cedri* is indeed *Wolbachia pipientis*. It should be stressed that an earlier molecular analyses did not detect *Wolbachia* in aphids (Tsuchida et al., 2002). However, recent extensive studies have indicated that this bacterium occurs sporadically in some populations of representatives of several aphid families (Wang et al., 2009; Augustinos et al., 2011).

Extensive, histological investigations by Buchner (1965) and more recent ultrastructural studies (Cheng & Hou, 2001; Szklarzewicz & Moskal, 2001; Szklarzewicz et al., 2006, 2010, 2013; Sacchi et al., 2008; Kuechler et al., 2010, 2011; Matsuura et al., 2012; Swiatoniowska et al., 2013) have shown that during hemipteran evolution various modes of inheritance of endosymbiotic microorganisms by subsequent generations have developed. The extracellular gut bacteria that are typical of most the heteropterans so far examined (i.e. members of families Pyrrhocoridae, Plataspididae, Acanthosomatidae, Reduviidae, Pentatomidae, Scutelleridae, Coreidae, Cydnidae, Parastrachiidae and several other families of Pentatomomorpha) are as a rule vertically – postnatally transmitted either by the contamination of the eggs with the symbionts, by deposition of a special capsule filled with symbionts onto the eggs or by feeding on the mother's excrements (see e.g. Buchner, 1965; Fukatsu & Hosokawa, 2002; Prado et al., 2006; Kaltenpoth et al., 2009; Kikuchi, 2009; Kaiwa et al., 2010; Hosokawa et al., 2010, 2013 for further details). In contrast, mycetomic bacteria of the other hemipterans (i.e. aphids, scale insects, whiteflies, psyllids, leafhoppers, planthoppers, coleorrhynchans and heteropterans belonging to families Cimicidae, Blissidae and Lygaeidae) invade female germ cells prenatally (i.e. in the ovaries of the mother) (see e.g. Buchner, 1965; Szklarzewicz & Moskal, 2001; Szklarzewicz et al., 2006, 2010, 2013; Sacchi et al., 2008; Kuechler et al., 2012, 2013; Swiatoniowska et al., 2013

for further details). Both the histological studies of Buchner (1965, 1966, 1967) and more recent ultrastructural studies (Cheng & Hou, 2001; Szklarzewicz & Moskal, 2001; Szklarzewicz et al., 2006, 2010, 2013; Sacchi et al., 2008; Kuechler et al., 2011, 2012; Koga et al., 2012; Matsuura et al., 2012; Swiatoniowska et al., 2013) have revealed that the latter transmission of endosymbionts (termed transovarial transmission) may differ even in closely related groups of hemipterans. The endosymbiotic microorganisms may invade young germ cells termed cystocytes (i.e. before differentiation into oocytes and trophocytes) or oocytes at the stage of choriogenesis (i.e. at the end of their growth). The ovaries may be invaded by whole intact bacteriocytes or by bacteria released from the cytoplasm of bacteriocytes. Within hemipterans scale insects exhibit the greatest diversity of modes of transovarial transmission of endosymbionts (Buchner, 1965, 1966, 1967; Szklarzewicz et al., 2006, 2010, 2013; Niznik & Szklarzewicz, 2007). Observations on the behaviour of endosymbiont in aphids (Buchner, 1965; Braendle et al., 2003; Miura et al., 2003; Wilkinson et al., 2003; Pyka-Fosciak & Szklarzewicz, 2008; Koga et al., 2012) have shown that these insects unlike other hemipterans are characterized by a rather uniform mode of transmission of microorganisms from mother to offspring. In all the viviparous females studied the bacteria invade embryos at the blastula stage. Recently, Koga and co-workers (2012) using electron microscopy and the FISH technique have shown that bacteria enter the embryo via exo/endocytotic transport, i.e. they are released from bacteriocytes and enter the embryonic syncytium by endocytosis. In oviparous females both primary as well as secondary endosymbionts invade ovaries containing terminal oocytes at the stage of advanced choriogenesis. In most species the bacteria migrate through the spaces between neighbouring follicular cells. Only in two species, *Clethrobius comes* (Drepanosiphidae) and *Schizolachnus pineti* (Lachnidae) do endosymbionts migrate both between neighbouring follicular cells and via the cytoplasm of these cells. In all species studied the endosymbionts enter the ooplasm and accumulate at the posterior pole of the oocyte forming a “symbiont ball”. It should be noted that Pyka-Fosciak & Szklarzewicz (2006, 2008) observed that small, rod-shaped bacteria (i.e. the third type of microorganism, see above) present in *Stomaphis quercus* (Lachnidae) are also maternally inherited but in contrast to the primary and secondary endosymbionts they invade larval ovaries containing undifferentiated germ cells (i.e. cystocytes). As a consequence, in the ovaries of an adult female these bacteria are present both in trophocytes and oocytes. This mode of transmission has never been observed in other aphids but is quite common in scale insects (Buchner, 1965; Niznik & Szklarzewicz, 2007; Szklarzewicz et al., 2010, 2013). In contrast to the situation encountered in *Stomaphis quercus*, the third type of microorganisms was not observed in germ cells of *Eulachnus rileyi*. This seems to indicate that these bacteria may be horizontally transmitted in this case.

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